

Protein crystals grow purer in space: physics of phenomena.

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This presentation will summarize the quantitative experimental and theoretical results obtained by B.R. Thomas, P.G. Vekilov, D.C. Carter, A.M. Holmes, W.K. Witherow and the Author, the team with expertise in physics, biochemistry, crystallography and engineering.

Impurities inhomogeneously trapped by a growing crystal - e.g., producing sectorial structure and/or striations - may induce macroscopic internal stress in it if an impurity molecule has slightly ($< 10\%$) different shape or volume than the regular one(s) they replace. We tested for the first time plasticity and measured Young modulus E of the triclinic, not cross-linked lysozyme by triple point bending technique. Triclinic lysozyme crystals are purely elastic with $E \approx 1 \div 5 \cdot 10^9 \text{ dyn/cm}^2$. The strength limit, $\sigma_c \approx 10^{-3} E \approx E \epsilon_c$, where σ_c and ϵ_c are critical stress and strain, respectively. Scaling E and σ_c with the lattice spacing suggests similar binding stiffness in inorganic and biomolecular crystals. The inhomogeneous internal stress may be resolved in these brittle crystals either by cracking or by creation of misoriented mosaic blocks during, not after growth. If each impurity molecule induces in the lattice elementary strain $\epsilon_0 \approx 3 \cdot 10^{-2}$ (this is maximal elementary strain that can arise at the supersaturation $\Delta\mu/kT \approx 2$) and macroscopic molecular concentration difference between subsequent macrolayers or growth sectors is $\delta C \approx 5 \cdot 10^{-3}$, the internal strain $\epsilon \approx \epsilon_0 \delta C \approx 10^{-4}$. Mosaic misorientation resolving such strain is ~ 30 arcsec. Tenfold increase of impurity concentration may cause cracking. Estimates of stress in an isometric sectorial crystal show that lysozyme crystals can tolerate the stress till the size of 0.5mm. Dissolving mosaic lysozyme crystal shows that the mosaicity, indeed, is absent below that size.

Crystallization of ferritin and lysozyme in space and on Earth was studied in presence of ribonuclease, insulin, cytochrome C, myoglobin, ovalbumin and dimers of both proteins, and with acetylated lysozyme on Earth. Crystals were dissolved to measure impurity distribution coefficients, K and k , defined as

$$K = (C_{is}/C_{ps})/(C_{il}/C_{pl}) = k(C_{pl}/C_{ps}). \quad (1)$$

Here C stands for concentration of impurity (i) and the crystallizing protein (p) in the solid (s) single crystal, and liquid (l) solution.

Lysozyme was acetylated by acetic anhydride. Acetylated form was purified by preparative HPLC and added as an impurity in the amounts $C_{il} = 0.76, 0.38, 0.1 \text{ mg/ml}$ to the solutions of the regular lysozyme, at $C_{pl} = 20, 30, 40 \text{ mg/ml}$, pH 4.5, with 50mM sodium acetate buffer and 4% of NaCl precipitant - to grow tetragonal crystals. Two crystallization runs were performed for each C_{il}, C_{pl} pair and 18 runs in total. All of the results are described by $K = 2.15 \pm 0.13$ and 3.42 ± 0.25 for the higher and lower degree of acetylation, respectively. These K are independent of both C_{il} and C_{pl} used. This independence is explained by constancy of the effective impurity adsorption and trapping rate, u_i , determining the impurity flux $C_{il}u_i$ to growing surface. Then $K = C_{il}u_i C_{pl}/C_{ps} \cdot V C_{il} \approx u_i/\beta$ where the crystal growth rate $V = \beta(C_{pl} - C_{pl}^{(e)})/C_{ps}$, $\beta \approx 5 \cdot 10^{-7} \text{ cm/s}$ is kinetic coefficient for lysozyme and $C_{pl}^{(e)} \approx 3 \text{ mg/ml} \ll C_{ps}$ is the lysozyme equilibrium concentration. Thus the law $C_{is} \propto 1/C_{pl}$ is in agreement with all the data and is equivalent the independence of K on C_{il} and C_{pl} .

Direct observations of ferritin dimers on the growing (111) face of ferritin crystals by AFM shows that practically all of these dimers are trapped by the propagating growth steps. We assume that the same is valid for lysozyme dimers and acetylated lysozyme molecules on the lysozyme growing faces. AFM visualize also lattice disturbance around trapped dimers.

Table 1 shows for the first time that space grown crystals contain several times less impurities than their terrestrial counterparts. We obtained similar results for lysozyme. This effect of self-purification in microgravity may be understood solving quasi steady state diffusion equation with the impurity balance at the growing interface, $r = R$: $D_i \partial C_{il}/\partial r = (k-1)C_{il}V$ where r is the distance from the crystal center, R is the effective crystal radius and D_i is diffusivity of the impurity. Within this approximation, impurity concentration at the interface decreases

proportional to $1/(1 + \beta_i R/D_i)$ as the crystal size R increases. Here $\beta_i = (k - 1)V \approx \beta K$ is the kinetic coefficient for the impurity trapping. The decrease happens just because $k = K(C_{ps}/C_{pl}) \gg 1$, i.e. impurity concentration per unit volume of the crystal is much larger than that of the solution. Thus the impurity concentration should decrease from the crystal core to the crystal periphery i.e. in the major crystal volume essential for diffraction. Correspondingly, effective distribution coefficient, K_{eff} , i.e. the one averaged over the whole crystal volume is:

$$K_{eff} = \langle K/(1 + \beta_i R/D_i) \rangle = (3D_i/\beta_i R)^3 \{[(\beta_i R/D_i)^2/2 - (\beta_i R/D_i) + \ln(1 + \beta_i R/D_i)]K + K\} \quad (2)$$

Table 1. Distribution Coefficients of Impurities in Ferritin

Monomer: $pI=4.0$, Charge: $(-)$, $M_r=480$ KDa, 24 Subunits, $M_r=19$ KDa;
Solution: Ferritin -1mg/ml, NaAc-0.2M, pH 5.0, CdSO₄-2.5% w/v; Impurity:
Dimer 0.05 mg/ml (5% w/v), Others-10% w/v

Impurity, M_r (KDa), pI , Charge	Gel		Space	
	K	k	K	k
Dimer	900	4.2-	4	300
Albumin	14	9.5+	0.1	9.9
Insulin	5.7	6.2+	0.02	2
Cytochrome C	13	10.5+	0.02	2
Myoglobin	17	7.0+	0.006	0.6
Ovalbumin	42	4.6-	$<10^{-2}$	$<10^{-2}$

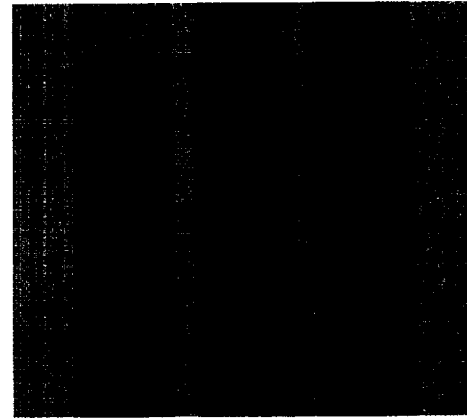


Fig. 1

Opposite to what happens in a stagnant solution, convection flow brings the impurity at a higher rate to the growing surface resulting in $K_{eff} \approx K$. For ferritin, the measured values are $\beta \approx 1.8 \cdot 10^{-5} \text{ cm/s}$, $V \approx \beta \omega C_{pl} \approx 4 \cdot 10^{-8} \text{ cm/s}$. For ferritin dimers, $\beta_i = 7.2 \cdot 10^{-5} \text{ cm/s}$, $D_i = 2.2 \cdot 10^{-7} \text{ cm}^2/\text{s}$, so that at $R = 100 \mu\text{m}$, $\beta_i R/D_i = 3.3$ and $K_{eff}/K = 0.3$ which, taking into account all approximation made, is in agreement with experimental findings. Lower impurity concentration around the crystal may result also in a higher growth rate and larger crystal size. If $k < 1$, the opposite effects of impurity enriched zone, $K_{eff} > K$, and smaller crystals may be expected. For nearly isomorphic impurities, like dimers, $K > 1$ and $\beta_i \approx \beta K > \beta$. Therefore depletion with respect to impurity around the growing crystal is several times deeper than that with respect to the crystallizing protein.

Fig. 1 obtained in collaboration with J.M. Garcia-Ruiz demonstrates impurity depletion zone surrounding apoferritin ($M = 450 \text{ KDa}$) crystals grown in gel - in absence of convection. We used iron containing and thus red colored holoferritin dimers as impurity to the colorless apoferritin. In the left tube, the meniscus separates the ferritin monomer and dimer containing gel (below) from the liquid solution of CdSO₄ precipitant (above). It is clear that the ferritin crystals are surrounded by the impurity depletion zone where the color provided by dimers is less intense. In the middle and right tubes no precipitant was added and no crystals appeared. In these tubes, diffusion levels the concentrations of dimers and trimers, respectively between the upper and lower parts of the tubes. Thus, the depletion in the left tube is due to the crystals.

To the best of our knowledge, the concept of the impurity depletion zone we develop provides first rational explanation of why and when protein crystals grown in microgravity may have better quality.

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Quantitative experimental and theoretical results on impurity trapping by growing protein crystals under terrestrial and microgravity conditions and on related lattice distortions obtained by B.R. Thomas, P.G. Vekilov, D.C. Carter, A.M. Holmes, W.K. Witherow and the Author will be summarized.

Triple point bending of triclinic lysozyme crystals show that they are purely elastic: internal stress may be eliminated during growth only. Young modulus, $E = (1 \text{ to } 5) \cdot 10^9 \text{ dyn/cm}^2$, the cracking stress $(10^{-3})E$. Several percent of impurity may induce internal stress sufficient for cracking.

Distribution coefficient, K , was measured for 9 impurities in ferritin and lysozyme. For acetylated lysozyme, K do not depend on the impurity or protein concentrations. AFM of ferritin dimer confirms microscopic features of trapping. Crystals grown in space are several times purer than with convection - because of impurity depletion zone. Together with J.M. Garcia-Ruiz, we visualized this zone around ferritin crystals in gel. Physical theories of crystal property scaling, impurity trapping and the microgravity effect explaining these experiments will be presented.